

AN EPIGENETIC LOOK AT ATRAZINE TOXICITY

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Abstract

Atrazine is a commonly used herbicide in the United States that is reported to contaminate drinking water sources. Studies indicate atrazine adversely impacts the neuroendocrine and reproductive systems, and that it may be a potential carcinogen. The current maximum contaminant level in drinking water is 3 parts per billion (ppb); however, levels higher than 3 ppb are often reported. Ongoing studies in our laboratory are investigating the immediate and latent adverse health outcomes associated with a developmental atrazine exposure and identifying the genetic and epigenetic mechanisms of toxicity using the zebrafish model system. MicroRNAs (miRNAs) are epigenetic regulators that posttranscriptionally control the translation of mRNA. A preliminary study indicated expression of miR-126, a miRNA associated with angiogenesis and tumorigenesis, to be altered following an embryonic atrazine exposure. To further investigate the developmental expression of miR-126 in zebrafish, quantitative PCR (qPCR) was used to profile expression throughout embryogenesis. Expression of miR-126 was developmental time point specific with significant peaks in expression at 36, 60, and 72 hours postfertilization (hpf). The deregulation of miR-126 by atrazine was also tested using qPCR. Zebrafish embryos exposed to 0.3, 3, or 30 ppb of atrazine were compared to a control treatment at all six developmental time points. While a dose-response trend in upregulation at 36 hpf and in down-regulation at 48 hpf was observed, a significant increase in expression was only observed at 60 hpf in embryos exposed to 30 ppb atrazine. This study is providing a greater understanding of an epigenetic mechanism of atrazine toxicity.

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Keywords

atrazine, embryogenesis, environmental exposure, epigenetics, herbicides, microRNA, miR-126, toxicology, zebrafish

Mentors

Jennifer L. Freeman is an associate professor of toxicology in the School of Health Sciences at Purdue University. Freeman received her PhD in environmental toxicology and molecular cytogenetics from the University of Illinois at Urbana-Champaign and was a postdoctoral fellow at Harvard Medical School and Brigham and Women's Hospital. Current research efforts in the Freeman laboratory are defining the underlying genetic and epigenetic mechanisms of a developmental exposure to environmental chemicals, including pesticides and metals, using the zebrafish vertebrate model system.



Sara E. Wirbisky received her master's degree in toxicology from Purdue University in August 2013. She received her undergraduate degree in general biology from Minnesota State University in 2010. Currently, she is completing her PhD at Purdue University and working in the laboratory of Dr. Jennifer Freeman.





AN EPIGENETIC LOOK AT ATRAZINE TOXICITY:

An Analysis of MicroRNA-126 Expression in Developing Zebrafish Exposed to the Herbicide Atrazine

Kelly E. Schlotman, General Health Sciences

INTRODUCTION

Atrazine is one of the most commonly used herbicides in the United States, especially in the Midwest. It is applied primarily to corn, but also to sorghum and sugarcane crops before and after their emergence. In 2013, six million acres of corn were planted in Indiana (U.S. Department of Agriculture, 2013). Using data obtained from the United States Census Bureau (2010), it can be calculated that about 26% of the total land in Indiana is dedicated to planting corn. Herbicides such as atrazine play a major role in the success rate of such large crop productions. The advantages of using herbicides are numerous: farmers apply them to reduce soil erosion, eliminate the manual removal of weeds, and increase crop production rates (Gianessi & Sankula, 2003). Due to its effectiveness and low cost, an estimated 76.4 million pounds of atrazine are applied each year in the United States primarily in the corn-belt region (U.S. Environmental Protection Agency, 2014a).

After application to crops, atrazine, a water-soluble chlorotriazine herbicide, persists in the environment and moves in soils. As a result, atrazine is often reported to contaminate drinking water sources (Adgate et al., 2001; Barr, Panuwet, Nguyen, Udunka, & Needham, 2007). Atrazine was banned in the European Union in 2004 because of widespread water contamination issues. In the United States, atrazine is regulated by the Environmental Protection Agency (EPA). The EPA set the maximum contaminant level (MCL) in drinking water at 3 parts per billion (ppb) in 1991 under the Safe Drinking Water Act; however, levels higher than 3 ppb are reported in drinking water in areas where atrazine is heavily used (U.S. Environmental Protection Agency, 2014b). The set MCL is intended to eliminate any adverse health effects

from chronic exposure (U.S. Environmental Protection Agency, 2014b).

Due to its prevalence in the environment, many studies have investigated atrazine's potential toxic and carcinogenic effects as a result of chronic and acute exposure. These studies indicate that atrazine is an endocrine disrupting chemical and suggest that it may have carcinogenic potential (Cragin et al., 2011; Cooper et al., 2007; Freeman et al., 2011; Hayes et al., 2010; reviewed in Hayes et al., 2011). Many epidemiological and laboratory studies are focused on atrazine exposure during development, as development is a life stage at which atrazine exposure may pose an increased risk for adverse health outcomes. These adverse effects may include birth defects, as was suggested in a study finding particular vulnerability to being born small for gestational age (SGA) after atrazine exposure in the third trimester of pregnancy (Villanueva, Durand, Coutté, Chevrier, & Cordier, 2005). In addition, a correlation between atrazine in drinking water and an increased risk of fetal growth impairment was reported in a study conducted on water sources in Indiana (Ochoa-Acuña, Frankenberger, Hahn, & Carbajo, 2009) and in a study on water sources in France (Chevrier et al., 2011). In a large-scale longitudinal study linking agrichemicals in the environment to birth defects, atrazine and other pesticides and nitrates were reported to be at their peak levels in the months of April through July. Newborns conceived during this time were observed to have a significantly higher risk of acquiring a birth defect. Newborns were at an increased risk for spina bifida and circulatory, tracheal, gastrointestinal, urogenital, and musculoskeletal anomalies as well as cleft

Figure 1 (Above). Adult zebrafish swimming in a tank.

lip, adactyly, clubfoot, and Down's syndrome (Winchester, Huskins, & Ying, 2009). The presence of limb birth defects such as adactyly and clubfoot also were reported at a higher incidence in an additional study in persons living in close proximity to cornfields rather than in persons living near soybean fields. The outcomes of this data suggest atrazine as a potential contributing factor to acquiring these birth anomalies due to its use primarily in corn but not in soybean production (Ochoa-Acuña & Carbajo, 2009).

Aside from epidemiological studies, atrazine also has been studied using several different animal models, including amphibians, fish, and rodents, in an effort to elucidate its effects on the human body. Although the specific manner in which atrazine acts is still unknown, studies report that atrazine functions in a nonreceptor-mediated manner to alter the release of prolactin and luteinizing hormone (Cooper et al., 2007; Roberge, Hakk, & Larsen, 2004; Tennant et al., 1994). These endocrine disrupting effects appear to be the cause of mammary gland tumor formation in female rats exposed to high doses of atrazine (Wetzel et al., 1994). Other studies reveal that atrazine may also alter CYP-450 aromatase activity (Heneweer, van den Berg, & Sanderson, 2004; Holloway, Anger, Crankshaw, Wu, & Foster, 2008; Sanderson, Seinen, Giesy, & van den

Berg, 2000; Suzawa & Ingraham, 2008). Disruption of this enzyme, which is involved in converting androgens to estrogens, has been the suggested cause for the observed demasculinization in male amphibians exposed to atrazine (Hayes et al., 2002). Current animal studies are attempting to identify additional genetic and molecular targets of atrazine exposure. In a study by Weber, Sepúlveda, Peterson, Lewis, and Freeman (2013), embryonic zebrafish exposure to low doses of atrazine resulted in significantly altered expression of genes associated with carcinogenesis, cell cycle function, and reproductive system development, function, and disease. This study also identified significant morphological alterations, including increased head length and head-to-body ratio.

In order to further define the mechanism of atrazine toxicity, in this study we used the zebrafish model system to begin to analyze the epigenetic changes that result from an embryonic atrazine exposure. Epigenetics is the study of the molecular mechanisms that regulate gene expression without changing the innate DNA sequence. According to Goldberg, Allis, and Bernstein (2007), epigenetics is the bridge between genotype and phenotype. The three categories of epigenetic mechanisms currently include noncoding RNAs, histone modifications, and DNA methylation. While a number of

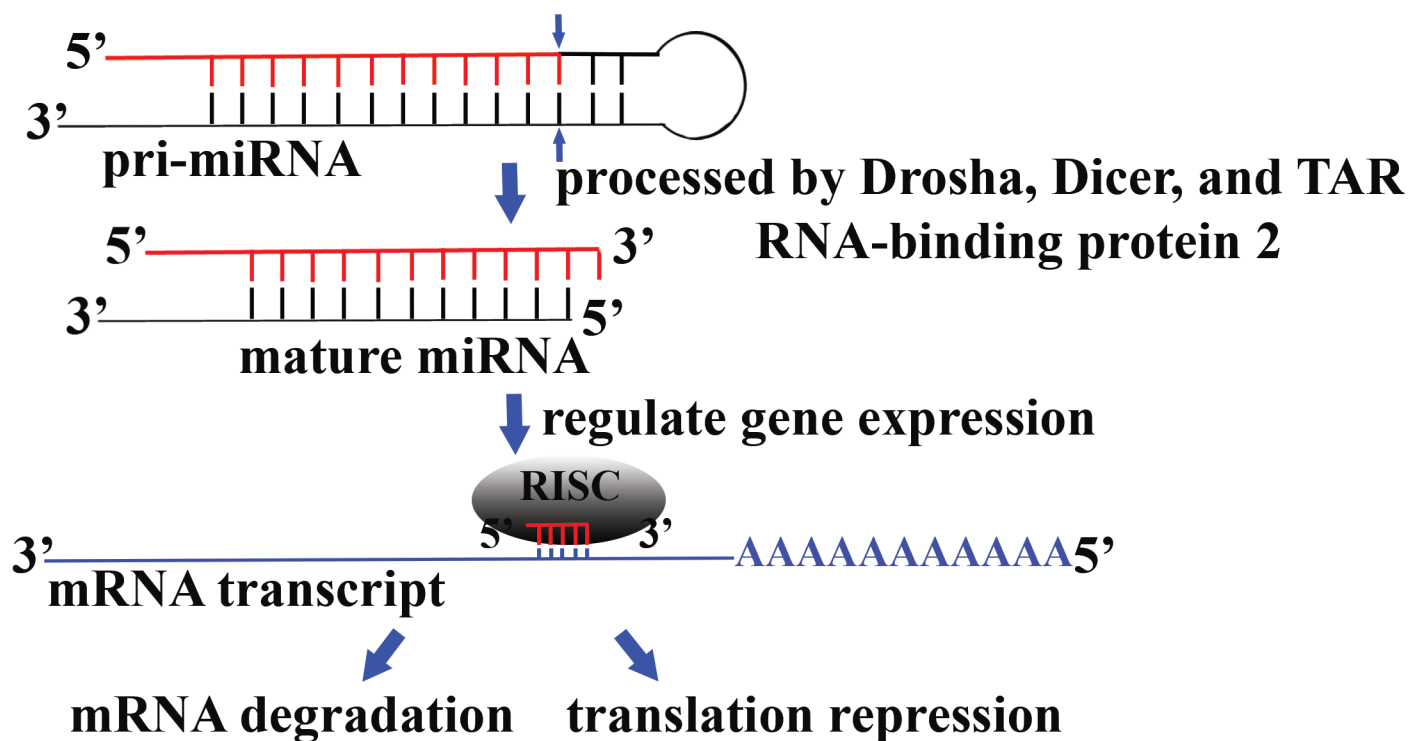


Figure 2. miRNA biogenesis and function. miRNA processing involves various specific enzymes such as Drosha, Dicer, and TAR RNA-binding protein 2. These enzymes function to modify primary miRNA (pri-miRNA) to produce a mature miRNA transcript. The mature miRNA combines with a specific protein from the Argonaute protein subfamily to form a RISC. Coupled together, they bind to the target mRNA sequence that is complementary to the mature miRNA sequence and induce mRNA degradation or block protein translation.

studies have focused on identifying alterations in DNA methylation associated with environmental chemical exposure, few studies have evaluated deregulation of microRNAs (miRNAs) (Freeman, Weber, & Sepúlveda, 2014). miRNAs are a class of small noncoding RNA that are approximately 22 nucleotides in length and are involved in a number of developmental processes. miRNA processing involves the enzymes Drosha, Dicer, and TAR RNA-binding protein 2 to produce the mature miRNA sequence (Figure 2). The mature miRNA combined with the RNA-induced silencing complex (RISC) can act to posttranscriptionally regulate messenger RNA (mRNA) by binding to the 3' untranslated region. Binding can result in mRNA degradation or translation repression of the mRNA transcript (Esteller, 2011).

miR-126 is the specific miRNA analyzed in this study, as preliminary data in our laboratory identified miR-126 to be a potential target of atrazine toxicity (data not shown). miR-126 is conserved among species and is found within intron 7 of the EGF-like domain-containing protein 7 (EGFL7) gene in all vertebrates (reviewed in Meister & Schmidt, 2010). miR-126 is found in highly vascularized tissues such as the heart, liver, and lung, and is the only miRNA known to be specifically expressed in the endothelial cell lineage, hematopoietic progenitor cells, and endothelial cell lines (reviewed in Meister & Schmidt, 2010). Past studies indicate that miR-126 is involved in regulating multiple cellular processes including blood cell development, inflammation, and angiogenesis (reviewed in Meister & Schmidt, 2010). miR-126 has a major role in vessel integrity and angiogenesis by targeting multiple different proposed gene transcripts in different mechanisms of action (Figure 3). In zebrafish, these targets include phosphoinositide-3-kinase regulatory subunit 2 (*pi3kr2*) and p21-activated kinase 1 (*pak1*). Each is involved in separate mechanisms of action with miR-126 (Sessa et al., 2012; Zou et al., 2011). Furthermore, miR-126 also enhances the expression of the vascular endothelial growth factor (*vegf*) by targeting Sprouty-related protein (*spred1*), and *pi3kr2* (Fish et al., 2008). Knockdown of miR-126 in zebrafish is associated with hemorrhage during embryogenesis, vascular remodeling, and maturation defects (Fish et al., 2008; Sessa et al., 2012; Zou et al., 2011). Down-regulation of miR-126 also is reported in cancerous tissue in the lung, stomach, cervix, bladder, and prostate (reviewed in Meister & Schmidt, 2010).

In this study, we utilized the strengths of the zebrafish model system to evaluate if an embryonic atrazine exposure would alter miR-126 expression. Zebrafish

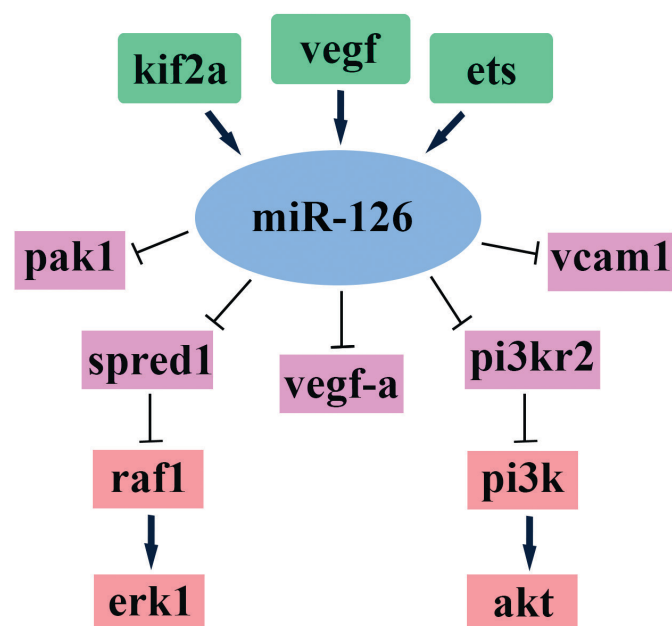


Figure 3. Targets of miR-126. miR-126 is proposed to target a number of different molecules involved in blood cell development, inflammation, and angiogenesis. Some of these targets include *pak1*, *spred1*, *vegfa*, *pi3kr2*, and *vcam1* (Fish et al., 2008; Sessa et al., 2012; Zou et al., 2011). *spred1* and *pi3kr2* both target negative regulators of the vegf pathway (*erk1* and *akt*, respectively).

have gained much popularity in many areas of biological and toxicological research (Hill, Teraoka, Heideman, & Peterson, 2005). They are small in size and produce large amounts of progeny at a time. Embryonic development is ex utero, and embryos can be easily exposed to a chemical of interest in a Petri dish or a 96-well plate and then visualized and assessed for abnormalities due to their optical clarity, transparent chorion, and well-defined developmental period. Furthermore, embryogenesis is complete in 72 hours. This rapid development allows for a short experimentation period to encompass all time points of embryonic development. In addition, the zebrafish genome is highly conserved with other vertebrate species, with 70% homology to humans allowing for translation from zebrafish studies to humans (Howe et al., 2013). The miR-126 sequence is conserved in zebrafish (Figure 4) and is reported to play a similar functional role in humans (reviewed in Meister & Schmidt, 2010), but there is limited information on the miR-126 expression profile during embryogenesis. The objectives of this study were then to first characterize expression of miR-126 throughout embryogenesis and to evaluate if an embryonic atrazine exposure resulted in deregulation of miR-126.

dre-miR-126a-3p ucguaccgugaguaauaauugc
dre-miR-126b-3p ucguaccgugaguaauaauugca
hsa-miR-126-3p ucguaccgugaguaauaauugcg

Figure 4. Zebrafish miR-126 sequence (dre-miR-126a-3p and dre-miR-126b-3p) and human miR-126 sequence (hsa-miR-126-3p) comparison. The zebrafish genome is currently reported to contain two copies of the miR-126 sequence termed dre-miR-126a and dre-miR-126b (Zou et al., 2011). Both zebrafish sequences are similar and identical to the human miR-126 sequence, with the exception of the last nucleotide. Sequences shown here are for miR-126-3p, as this was the target of the primer used in this study.

MATERIALS AND METHODS

Zebrafish Husbandry

The zebrafish used in this study were of the wild type AB strain and maintained in a Z-Mod System (Aquatic Habitats) set on a 14:10 hour light-dark cycle at 28°C. The water circulating in this system was checked two times per day to keep the pH between 7.0 to 7.2 and the salt concentration around 500 µS. Protocol with regards to feeding, breeding, and maintaining the zebrafish population was based on Westerfield's *The Zebrafish Book* (2007) and were approved by Purdue University's Institutional Animal Care and Use Committee. As zebrafish mating is stimulated by sunrise, adult zebrafish were bred in the morning using spawning tanks, and embryos were collected shortly after fertilization to use for experimentation.

Embryonic Developmental Time Course Collection

Embryos were collected, randomly separated into Petri dishes in groups of 50 (considered as subsamples), and allowed to develop until they reached a specified developmental time point. Developmental time points analyzed included 12, 24, 36, 48, 60, and 72 hours postfertilization (hpf) to encompass the entire embryonic period. Upon reaching the specified developmental time point, zebrafish in each Petri dish were collected and pooled to count as one biological replicate. Embryos and larvae were anesthetized in Tricaine (Sigma-Aldrich) and homogenized in Trizol Reagent (Life Technologies). Samples were flash frozen in liquid nitrogen and stored at -80°C until miRNA isolation. Four biological replicates were collected per time point to profile miR-126 throughout development.

Embryonic Atrazine Exposure

A stock solution of technical-grade atrazine (CAS 1912-24-9; Chem Service) was prepared at a concentration of 10 parts per million (ppm) near the solubility limit and was then diluted to make 0.3, 3, and 30 ppb atrazine solutions as outlined in Freeman and Rayburn (2005). These concentrations cover a range of exposures that are 0.1x to 10x the MCL currently set by the EPA. After the adult zebrafish breeding, embryos were collected, randomly separated into Petri dishes in groups of 50 (considered as subsamples), and allowed to develop until they reached a specified developmental time point. Time points analyzed spanned the zebrafish embryonic period and included 12, 24, 36, 48, 60, and 72 hpf. Upon reaching the specified developmental time point, zebrafish in each Petri dish were collected and pooled to count as one biological replicate. Embryos and larvae were anesthetized in Tricaine (Sigma-Aldrich) and homogenized in Trizol Reagent (Life Technologies). Samples were flash frozen in liquid nitrogen and stored at -80°C until miRNA isolation. Three biological replicates were collected per time point for each atrazine treatment to assess miR-126 expression alterations as a result of atrazine exposure.

miRNA Extraction and cDNA Synthesis

miRNA isolation was completed following manufacturer recommendations with the miRNeasy Mini Kit (Qiagen). The Nanodrop-1000 was used to check the quality of extracted miRNA. Isolated miRNA samples were used to make 5 µg miRNA samples, which were converted to cDNA using the Universal RT microRNA PCR kit (Exiqon) according to manufacturer protocols.

Real-Time Quantitative PCR

Gene expression was normalized to the reference primer U6. U6 and miR-126 (hsa-miR-126-3p; Figure 4) primers were designed and synthesized using locked nucleic acid technology by Exiqon. A BioRad CFX Connect Real-Time PCR Detection System was used with the SYBR Green Master Mix Universal RT according to manufacturer recommendations (Exiqon). Three technical replications were included for each sample in a plate. A dissociation curve was included to check for nonspecific amplification. Efficiency and specificity were checked with melting and dilution curve analysis and no-template controls. Quantitative copies were calculated by standard curve, and individual gene expression was normalized to U6 (ratio of miR-126/U6). The overall procedure is summarized in Figure 5.

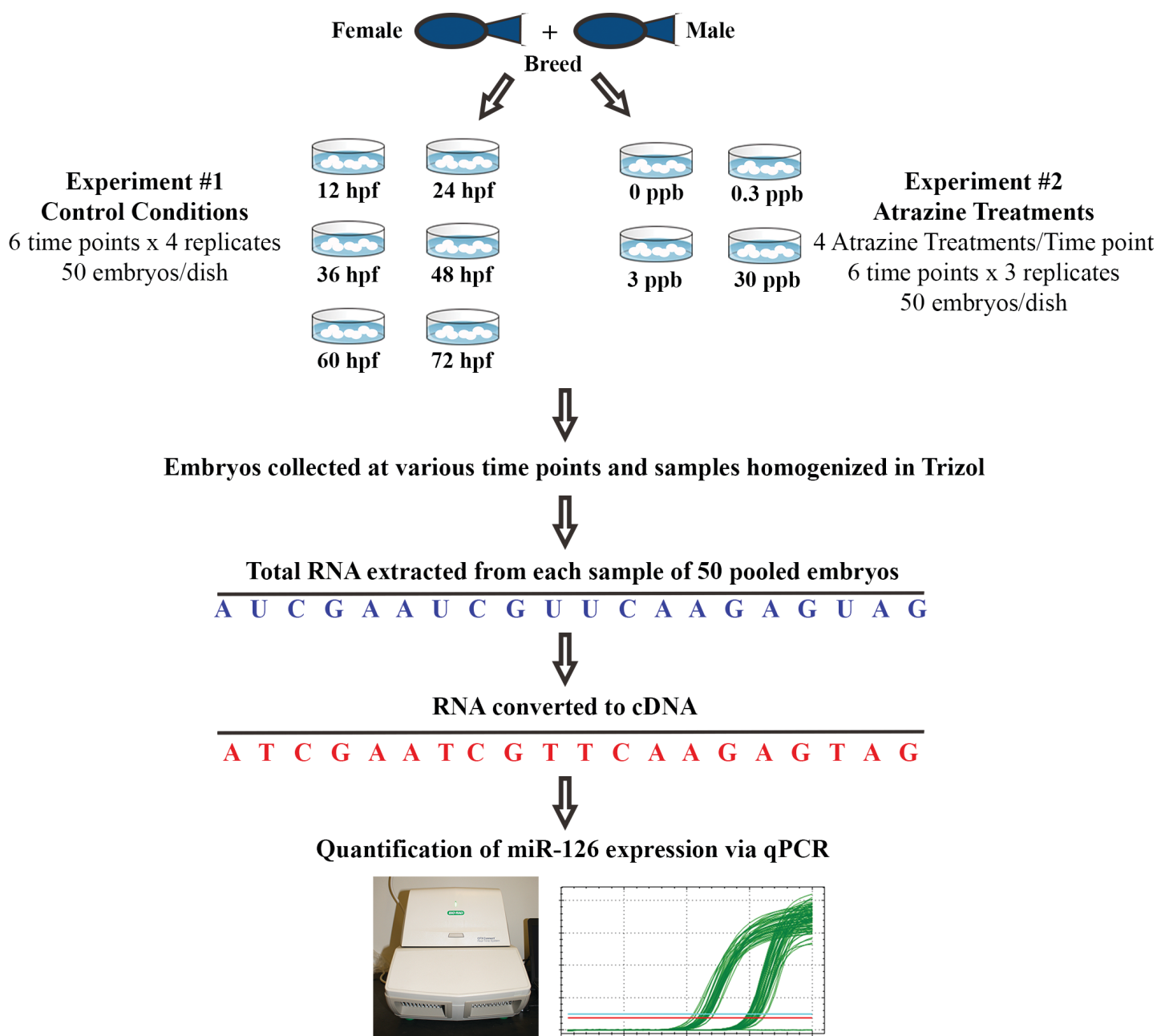


Figure 5. Quantitative PCR procedure. The first experiment involved profiling miR-126 expression throughout development at 12, 24, 36, 48, 60, and 72 hpf in control conditions. Four biological replicates ($n=4$) per time point were included in this analysis. The second experiment involved analyzing miR-126 expression after exposure to 0, 0.3, 3, or 30 ppb atrazine through each of the six developmental time points mentioned above. Upon reaching the specified developmental time point, embryos were collected and stored in Trizol for each experiment. When samples from all developmental time points were obtained, embryos were analyzed for miR-126 expression. This process involves total RNA extraction, cDNA synthesis, preparation of 0.0125 ng/ μ l cDNA stocks, and normalization of miR-126 expression with a reference (U6). Time points were analyzed using an ANOVA with a post-hoc LSD test when a significant ANOVA was observed ($p < 0.05$).

Statistical Analysis

The developmental time course and atrazine-treated samples were analyzed for statistical differences with a one-way analysis of variance (ANOVA). A post-hoc least significant difference (LSD) test was completed when a significant ANOVA was observed ($p < 0.05$).

RESULTS

miR-126 Developmental Time Course Expression Profile

qPCR was used to quantify miR-126 expression at six developmental time points under control conditions during embryogenesis. Results revealed that miR-126 expression is developmental time point specific (Figure 6). Expression at the 12 hpf time point was significantly different from all other time points and revealed very low expression. After 12 hpf, miR-126 expression increased and reached a peak level of expression at 36 hpf. After decreasing at 48 hpf, expression returned to higher levels at 60 and 72 hpf. Overall, 24 and 48 hpf showed statistically similar medial expression, while 36, 60, and 72 hpf were statistically similar with higher expression levels.

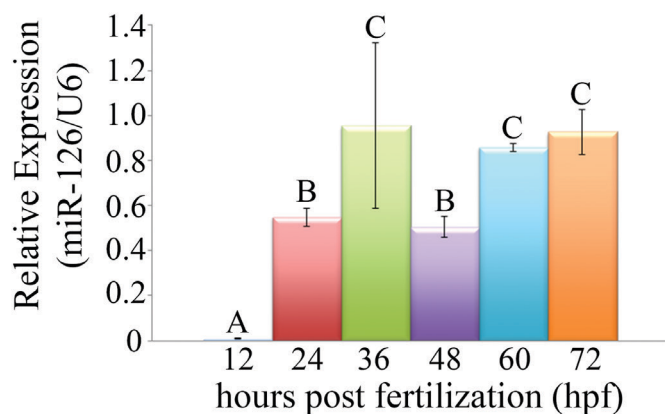


Figure 6. miR-126 expression throughout zebrafish development. Developmental time points with differing letters (A, B, or C) were found to be significantly different from one another ($p < 0.05$). miR-126 dramatically increased after 12 hpf and reached a peak expression at 36 hpf. After decreasing at 48 hpf, expression returned to higher levels at 60 and 72 hpf. miR-126 expression was normalized using U6 as a reference. Error bars depict standard deviation.

miR-126 Expression Following Atrazine Exposure at 12, 24, 36, 48, 60, and 72 hpf

qPCR was used to compare expression levels of miR-126 between control and atrazine-treated zebrafish at different developmental time points. Expression was minimal at 12 hpf for embryos in the control and atrazine treatments

(data not shown). No significant differences were observed between embryos in the control and atrazine treatments at 24 hpf (Figure 7A, $p = 0.1497$). A dose response trend in upregulation of miR-126 was observed at 36 hpf but was not statistically significant (Figure 7B, $p = 0.2557$). Similarly, a dose response trend in down-regulation of miR-126 was observed at 48 hpf, but was not statistically significant (Figure 7C, $p = 0.0998$). At 60 hpf, a significant increase in miR-126 expression was observed for embryos in the 30 ppb atrazine treatment (Figure 7D, $p = 0.0281$), but no significant difference was observed at 72 hpf (Figure 7E, $p = 0.5844$).

DISCUSSION

The expression of miR-126 during development and its potential alteration during a developmental atrazine exposure was analyzed using the zebrafish model system. Using qPCR to quantify expression at different developmental time points, a profile of miR-126 expression during zebrafish embryogenesis was first completed. miR-126 expression was developmental time point specific, with an increase in expression following 12 hpf and relatively high levels of expression occurring at 36, 60, and 72 hpf. Zou and colleagues (2011) also analyzed miR-126 expression throughout zebrafish embryogenesis but with a transgenic zebrafish line. Their experiment included 12, 24, 36, 48, and 72 hpf time points and revealed increasing expression as development progressed; however, no analysis was performed to examine if expression at each time point were statistically significant from one another. While findings in the Zou and colleagues (2011) study and the present study both find that miR-126 expression increases after 12 hpf, they differ in their analysis at 48 hpf. This difference may be attributed to the use of differing zebrafish lines or sample sizes. The current study used the wild type AB zebrafish strain, while Zou and colleagues (2011) used a transgenic line. Thus, genetic profiles of the zebrafish are different and may also result in a slightly different maturation progression. In addition, it appears Zou and colleagues (2011) sampled three single embryos at each time point to equal three biological replicates. In the current study, 50 zebrafish embryos were pooled at each time point for each of the four biological replicates to reduce genetic variation that may be present when comparing single fish. As a result, 200 total embryos were sampled at each developmental time point.

After fertilization, zebrafish embryos undergo rapid changes in morphology (Figure 8). Due to miR-126's known involvement with angiogenesis and vascular integrity, the variation in expression seen in this study is hypothesized to be linked to the rapid vascular development occurring during zebrafish embryogenesis.

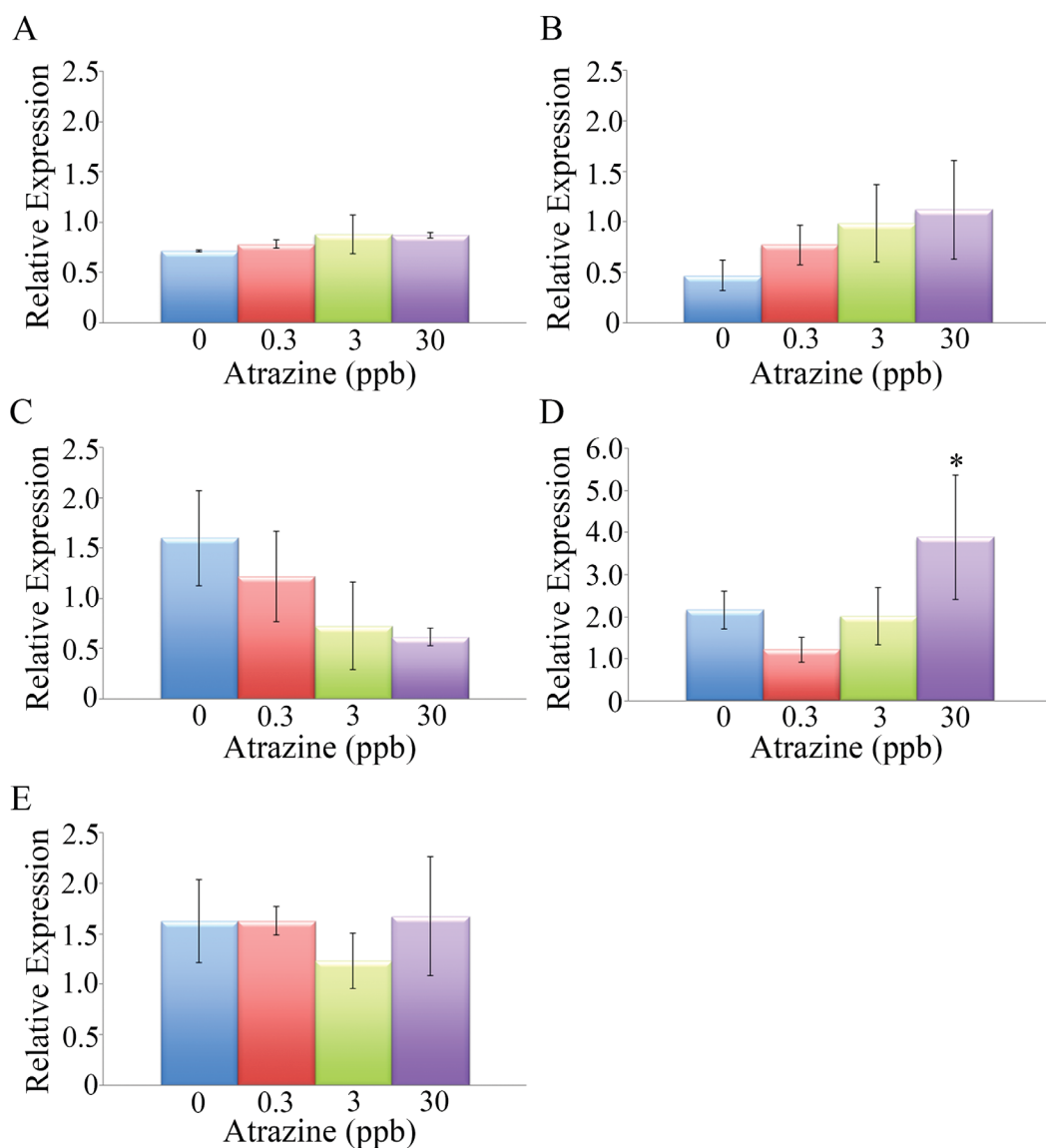


Figure 7. Altered miR-126 expression throughout embryogenesis following atrazine treatment. miR-126 expression was examined at six developmental time points following exposure to one of three atrazine treatments (0.3, 3, or 30 ppb) and compared to a control treatment. Minimal expression was observed at 12 hpf (data not shown). No significant difference in expression was observed at 24 (A), 36 (B), or 48 hpf (C), although an increasing dose trend was observed at 36 hpf, and a decreasing dose trend was observed at 48 hpf. Expression levels were significantly altered in embryos treated with 30 ppb atrazine through 60 hpf (D). No significant difference was observed at 72 hpf (E). miR-126 expression was normalized using U6 as a reference. (* $p < 0.05$, atrazine treatment to control). Error bars depict standard deviation.

Angiogenesis describes the process by which new vessels are formed from existing vessels. In early zebrafish development, this process occurs in two surges to form the intersegmental vessels (ISVs). The first wave begins at 20–24 hpf, with the formation of the first ISVs from the dorsal aorta (DA) (reviewed in Ellertsdóttir et al., 2010). Around 30 hpf, each new sprout formed from the DA is made up of 3 to 4 cells with distinct positional fates (reviewed in Baldessari & Mione, 2008). The differentiated functions of these cells allow each ISV to grow dorsally to form segmental arteries (SA) and eventually connect with neighboring ISVs to form the dorsal longitudinal anastomotic vessel (DLAV) (reviewed in Ellertsdóttir et al., 2010). At 32–34 hpf, the second wave of angiogenesis occurs with the formation of small branches from the posterior cardinal vein (PCV) (reviewed in Ellertsdóttir et al., 2010). These new vessels merge with existing SAs to form segmental veins (SV) or

grow separately to contribute to the lymphatic vasculature (reviewed in Ellertsdóttir et al., 2010).

In the current study, miR-126 was found to have minimal expression at 12 hpf and then increase in expression at 24 hpf corresponding to the first wave of angiogenesis. In addition, an increase in miR-126 was observed at 36 hpf, suggesting relation to the second wave of angiogenesis. Furthermore, a study by Nicoli and colleagues (2010) analyzed angiogenesis of the accessory fifth aortic arch (AA5x), which occurs around 60 hpf, and its dependence on blood flow. Results revealed that the flow-dependent transcription factor, *krueppel-like factor 2* (*klf2a*), was responsible for activating miR-126 to induce *veg*f signaling and allow for the formation of a patent circulatory connection (Nicoli et al., 2010). The increase in miR-126 expression later in zebrafish development at 60 and 72 hpf in our study suggests a relationship to this mechanism.

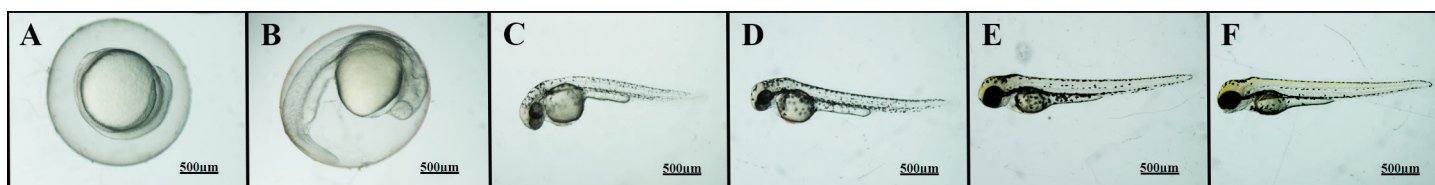


Figure 8. Zebrafish developmental stages. Embryogenesis in the zebrafish is classified as the first 72 hours of life. During this time, zebrafish undergo rapid morphological changes. Developmental progression is based on temperature with the following descriptions based on the staging series at 28°C (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995). At 12 hpf (A), the first 5–6 somites appear and the neural plate is present. By 24 hpf (B), the heart has formed into a cone-shaped tube, and pigment formation begins in the retinal epithelium and in the melanophores dorsolaterally in the skin. By 36 hpf (C), a single pair of aortic arches has formed. Zebrafish embryos generally hatch from their chorion sometime between 36–72 hpf. Hatching times vary, and hatching does not significantly indicate a developmental stage of maturation. By 48 hpf (D), the dorsal strip of melanophores is well defined, the heart beats, and all aortic arches are present. At 60 hpf (E), pigmentation of the retina is dense, the heart beats strongly and is full of circulating blood, and blood flows in all of the aortic arches. At 72 hpf (F), the yolk sac is diminishing and embryogenesis is complete.

miR-126 has been observed to be significantly altered in other toxicological studies with zebrafish embryos. For example, Zhao, Xiong, and Xie (2011) reported altered expression of miR-126 in response to a developmental microcystin exposure. The deregulation of miR-126 was attributed with the loss of vascular integrity observed in the zebrafish embryos at 72 hpf (Zhao et al., 2011). In the present study, miR-126 deregulation was not observed in the majority of the developmental time points. The 30 ppb atrazine-treated embryos through 60 hpf were the only cohort to show significantly altered expression. While atrazine does not appear to have an effect on ISV formation due to the lack of deregulation at 24 and 36 hpf, the deregulation at 60 hpf may have an effect on the mechanism governing angiogenesis in the aortic arches, specifically AA5x. As mentioned above, angiogenesis during AA5x development is induced by blood flow, which activates *klf2a* to induce miR-126, which results in enhanced *vegf* expression (Nicoli et al., 2010).

In summary, the results of this study indicate that the expression of miR-126 is developmental time point specific with significantly high expression levels at 36, 60, and 72 hpf, medial expression at 24 and 48 hpf, and a very low expression at 12 hpf. The increased levels after 12 hpf and very high levels at 36, 60, and 72 hpf time points correspond to surges in angiogenesis during zebrafish development as ISV formation is known to occur around 20–24 hpf and 32–34 hpf, and angiogenesis in AA5x around 60 hpf (reviewed in Ellertsdóttir et al., 2010; Nicoli et al., 2010). Atrazine was found to alter expression of miR-126 only in embryos exposed to 30 ppb through 60 hpf. Alterations at this time point indicate that atrazine might alter angiogenesis corresponding with AA5x, but additional studies are needed to explore this hypothesis.

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